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TERMINI GENERATED AT THE SITE OF THE DNA BREAKAGE MEDIATED BY PHOTOEXCITED PROMAZINES

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Abstract—Promazine derivatives are known to be able to photoinduce, *in vitro*, direct single-strand breaks into DNA (Decuyper *et al.*, *Biochem. Pharmac.* **33**, 4025–4031 (1984)). Using [^{32}P]end labeled DNA fragments, it is demonstrated that this DNA breakage occurs almost regardless of the nucleotide sequence of the DNA. Using 3′-[^{32}P]end or 5′-[^{32}P]end labeled oligonucleotide and enzymatic digestion of the fragments generated, it is demonstrated that the termini generated at the site of the breakage are 5′-phosphate, 3′-phosphate and 3′-termini which are presumed to be 3′-phosphoglycolate. This is consistent with an attack of the sugar moeity of the sugar-phosphate backbone of the DNA by the reactives species generated upon near-u.v. irradiation of promazine derivatives.

Promazine derivatives (PZD)§ are well known to be active photosensitizers inducing various photodamages into biomolecules ([1] and references therein). DNA is an important target for the "PZD + near u.v. light treatment" of living systems. On one hand, the DNA bases can be altered. PZD have been shown to be able to form covalent photoadducts to these bases, mainly on guanines, which are termination sites for the DNA replication [2]. These adducts could be responsible for the photokilling effect of these drugs, observed in bacteriophages [3–5], in viruses [6], in bacterial cells [7] and in mammalian cells [8]. Nevertheless, these base modifications could also be responsible for mutation induction as proposed by Piette and Van de Vorst [9]. On the other hand, the sugar-phosphate backbone can also be altered by the "PZD + near u.v. light treatment". Fujita *et al.* [4] have detected alkali-labile bond formation and we have demonstrated that PZD can photosensitize true single-strand break induction [10]. This DNA breakage was shown to be due to hydroxyl radical formation upon near u.v. light irradiation of PZD in the presence of DNA. CPZ was shown to act mainly by promazinyl and/or chlorine radicals formation and MTPZ cation radical was detected to be an active DNA breaker. Nevertheless, the site of DNA attack responsible for the DNA nicking and the products of the reaction are unknown. The knowledge of the site of the DNA breakage and the chemical nature of the termini generated at the site of this cleavage are important with a view to understanding the *in vivo* repair mechanisms.

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§ Abbreviations used: CIP, calf intestine phosphatase; CPZ, chlorpromazine; PNK, T4-polynucleotide kinase; PZ, promazine; PZD, promazine derivatives; MTPZ, methoxypromazine; TFPZ, triflupromazine.

End labeled DNA fragments of known nucleotide sequence were irradiated in the presence of PZD and the sites of DNA breakage were determined using sequencing techniques [11, 12]. The determination of the natures of termini generated was based on the variation of electrophoretic mobility of the DNA fragments, generated by the reaction, versus their termini as described by Henner *et al.* [13–15].

EXPERIMENTAL PROCEDURE

Materials

PZD (from Specia, Paris, France; except TFPZ which was from M.S. Chemicals, Milano, Italy) were used as received, without further purification. γ-[^{32}P]ATP (3000 Ci/mmole) and α-[^{32}P]ddATP (3000 Ci/mmole) were from Amersham Corp. All enzymes were from Boehringer (Mannheim, F.R.G.). Oligo $(dA)_{12}$ and poly (dT) were from P-L Biochemicals (Uppsala, Sweden). Plasmid pDR540 (P-L Biochemicals, Uppsala, Sweden) has been amplified in *E. coli* K12 JM103, extracted and purified according to Maniatis *et al.* [16].

[^{32}P]*5′-end labeling of pDR540 DNA fragment*

pDR540 DNA was cleaved by EcoRI and BamHI, which restrict out pDR540 DNA a 500 base pairs DNA fragment. This fragment (80 pmoles) was dephosphorylated by treatment, at 37° for 60 min, with Calf Intestine Phosphatase (CIP, 10 units) in a buffer containing 50 mM Tris–HCl, pH 8.0 and 0.1 mM EDTA, the reaction volume was 40 μl. The reaction was stopped by adding 20 μl of CIP-stop mixture (20 mM Tris–HCl, pH 8.0, 1% SDS, 200 mM NaCl and 2 mM EDTA) and heating at 68° for 15 min. The mixture was then extracted twice with phenol and three times with ether and nucleic

acids were ethanol precipitated. The dephosphorylated fragment was resuspended into 50 µl containing T4-polynucleotide kinase (PNK, 50 units), γ-[^{32}P]ATP (80 pmoles), 50 mM Tris–HCl, pH 8.0, 10 mM $MgCl_2$, 5 mM DTT, 0.1 mM spermidine, 0.1 mM EDTA, 0.1 M KCl and 25 µg/ml BSA. The phosphorylating reaction was allowed for 60 min at 37°. The mixture was treated as described above and nucleic acids were ethanol precipitated. The labeled fragment was then cleaved using HindIII restriction endonuclease and the resulting 92 base pairs DNA fragment labeled at a single terminus was separated from the 408 base pairs DNA fragment by preparative polyacrylamide gel electrophoresis under denaturating conditions.

[^{32}P]5′-end labeling of oligo $(dA)_{12}$

5′-Dephosphorylation of oligo $(dA)_{12}$ was carried out as described above except that after reaction the mixtures were phenol extracted twice, ether extracted three times, spun filtrated on Sephadex G10 [16] and then lyophilized. 5′-End labeling was carried out as described above except that after the reaction, the mixtures were extracted as described above, spun filtrated on Sephadex G10, equilibrated with water [16] and then lyophilized. Labeled oligo $(dA)_{12}$ was purified by preparative polyacrylamide gel electrophoresis in denaturating conditions.

[^{32}P]3′-end labeling of oligo $(dA)_{12}$

3′-End labeling of oligo $(dA)_{12}$ (80 pmoles) was carried out using α-[^{32}P]ddATP (250 µCi, 80 pmoles) in the presence of terminal deoxynucleotidyl transferase (25 units) in a 50 µl volume containing 140 mM potassium cacodylate, 30 mM Tris–HCl, 1 mM $CoCl_2$, 0.1 mM DTT, pH 7.6. Mixtures were incubated for 2 hr at 37°. After reaction, the mixtures were treated as described above. The 3′-end labeled oligo $(dA)_{13}$ was purified as described above.

Photoreaction conditions

Various mixtures (60 µl total volume) were prepared. They contained: PZD (1 mM except when CPZ was used as sensitizer, 0.1 mM), 10 mM Tris–HCl (pH 7.5) and (i) the 5′-end labeled oligo $(dA)_{12}$ supplemented with 1 µg poly (dT) or (ii) the 3′-end labeled oligo $(dA)_{13}$ supplemented with 1 µg poly (dT) or (iii) the 92 base pair HindIII-EcoRI (from pDR540, 5′-end labeled). The mixtures were then irradiated using a high pressure vapor mercury lamp (Osram HBO 500, except when CPZ was used as sensitizer, xenon lamp XBO 150, Osram). The lamps were equipped with a WG 305 filter ($\lambda > 290$ nm, Schott, F.R.G.). After 4 hr irradiation (20 min when CPZ was used), the mixtures were extracted as described above and DNA was ethanol precipitated (oligonucleotide were recovered by lyophilisation after spun filtration on Sephadex G10) [16].

Polyacrylamide gel electrophoresis

Before loading on the gels, photoreacted material was resuspended into 5 µl of loading buffer (98% deionized formamide, 0.25% bromophenol-blue, 0.25% xylene-cyanol and 2% of 1 M Tris, 1 M boric acid and 5 mM EDTA). The samples were denaturated by heating for 3 min at 100° and chilling on an ice bath. The mixtures were then analysed by denaturating polyacrylamide gel electrophoresis (40 cm long, 0.8 mm thick, 7 M urea, 20% acrylamide). The gels were run at constant power (60 W). Autoradiography of the gels was carried out using Fuji-X-ray films.

Sequencing of DNA fragment

[^{32}P]5′-end labeled DNA fragment was sequenced according to the Maxam and Gilbert [11] procedure except for the T reaction which was from Rubin and Schmid [12].

Enzyme reactions

Fragments of photoreacted oligonucleotide were cut out from the gel, eluted overnight at 37° with 200 µl of elution buffer (0.5 M NH_4Ac, 1 mM EDTA), spun filtrated on Sephadex G10 [16] and then lyophilized. The fragments were resuspended into 15 µl water, 5 µl of which were lyophilized and kept as reference, the remaining 10 µl being enzymatically treated as described below.

CIP digestion. The 10 µl solution of fragments were diluted into 100 µl containing CIP (10 units), 50 mM Tris–HCl pH 8.0 and 0.1 mM EDTA. The reaction was allowed for 60 min at 37°. The reaction was stopped as described above. The mixture was extracted as described above, spun filtrated on Sephadex G10 [16] and then lyophilized. Digested and undigested fragments were then analysed by polyacrylamide gel electrophoresis in denaturating conditions.

ExoIII treatment. The 10 µl solution of fragments were diluted into 20 µl containing ExoIII (20 units), 30 mM Tris–HCl pH 7.5, 16 mM NaCl, 16 mM $MgCl_2$ and 2 µg poly (dT). The reaction was allowed for 5 min at 37°. The mixtures were then treated and analysed as described above.

T4-polynucleotide kinase (3′-phosphatase) treatment. The 10 µl solution of fragment were diluted into 50 µl containing PNK (10 units), 100 mM Tris–HCl pH 6.5, 10 mM $MgCl_2$ and 5 mM β-mercaptoethanol. The mixtures were incubated overnight at 37°. The mixtures were then treated and analysed as described above.

RESULTS

Sites of DNA breakage induced by photoexcited PZD

In a previous work [10], we have demonstrated that photoexcited PZD were able to induce single-strand breaks in DNA. This conclusion was based on the observation that photoexcited PZD were able to promote the conversion of the superhelical replicative form of ØX174 DNA into its relaxed form. This conversion was detected by agarose gel electrophoresis analysis, and thus it was not possible to determine if this DNA breakage occurred with a specificity depending on nucleotide sequence. Experiments were thus carried out in order to investigate this possibility.

The 92 base pairs DNA fragment (EcoRI-HindIII) of plasmid pDR540 labeled at its 5′-end was photoreacted in the presence of PZD and the products of

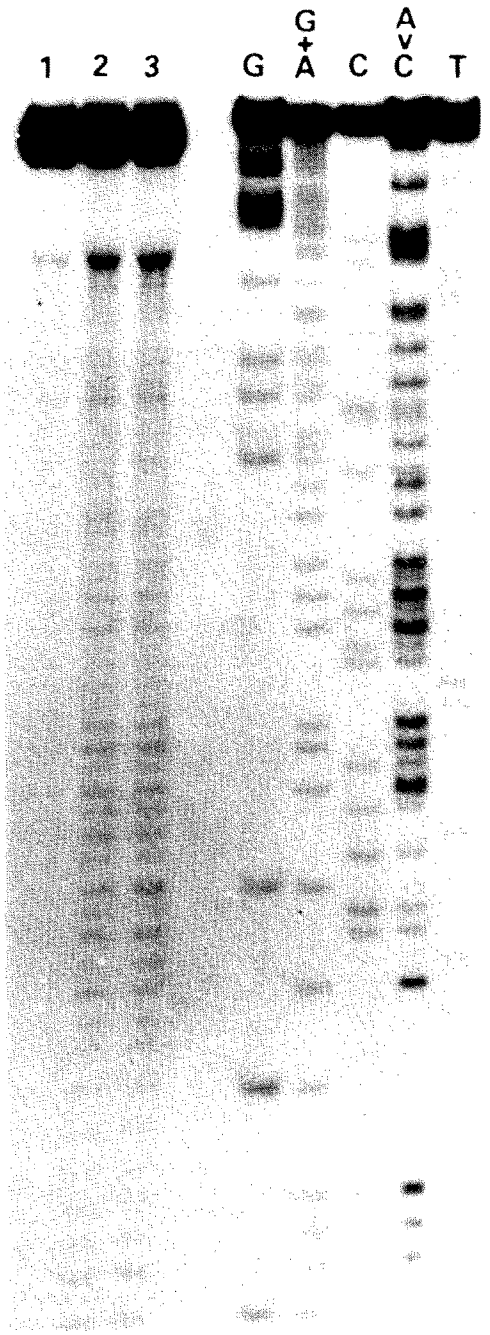

Fig. 1. Sites of DNA breakage induced by CPZ + near u.v. light treatment. The 92 base pairs HindIII-EcoRI 5′-end labeled pDR540 fragment was irradiated using a xenon lamp (XBO 150) equipped with a WG305 Schott filter (290 nm). After various irradiation times, samples were analysed by polyacrylamide (10%) gel electrophoresis in denaturating conditions. Lane 1 (1 min), lane 2 (3 min) and lane 3 (10 min). Lanes G, G + A, C, A C and T refer to the sequencing reactions.

the reaction were analysed by polyacrylamide gel electrophoresis in denaturating conditions. In order to map precisely the position of the DNA breakage in the nucleotide sequence, the product of sequencing done by chemical cleavage [11, 12] was run alongside the photoreaction product. Figure 1 shows the results obtained when CPZ was used as photosensitizer. PZ, TFPZ and MTPZ have led to similar results. DNA photoreactions with PZD lead to DNA breakage at each nucleotide. Nevertheless, it should be pointed out that the bands, corresponding to breakage at the level of purine residues, present intensities which are weakly higher than the other. Thus, "PZD + near u.v. light treatment" of the DNA promotes DNA breakage almost regardless of the nucleotide sequence.

5′-Termini generated by PZD photoreaction

DNA breakage induced by PZD photoreaction leads to the generation of DNA fragments. In order to determine the nature of the chemical structure of 5′-termini of these fragments, 3′-end labeled oligo $(dA)_{13}$ was irradiated in the presence of PZD and the reaction products were separated by polyacrylamide gel electrophoresis (20% acrylamide) under denaturating conditions. This method provides a high resolution separation of the fragments based on their length and the chemical structure of their 5′-end. Figure 2A shows the results obtained. DNA nicks occurred at each nucleotide, according to the results obtained above, and, in addition, only single bands were detectable at each site. Thus, one type of 5′-terminus is generated during the reaction.

In order to determine unambiguously the nature of these 5′-termini, the radioactive bands corresponding to the 6 nucleotide long fragment have been eluted from the gel and aliquots were digested with CIP. After this treatment, the electrophoretic mobilities of digested and undigested fragments were compared on denaturating polyacrylamide gel. Figure 2B shows the results obtained. Digestion with CIP leads to a decrease of electrophoretic mobilities of the fragments. This modification can be explained by the fact that CIP treatment leads to the transformation of a 5′-phosphate terminus into a 5′-hydroxyl terminus. Thus, DNA breakage induced via PZD photoreaction appears to promote, mainly, the generation of 5′-termini which are 5′-phosphate.

3′-Termini generated by PZD photoreaction

In order to determine the nature of the 3′-termini generated during PZD photoreaction, 5′-end labeled oligo $(dA)_{12}$ was irradiated in the presence of PZD. The products of reactions were separated by polyacrylamide gel electrophoresis in denaturating conditions. Figure 3A gives the results obtained. As above, it can be observed that DNA breakage occurred at each nucleotide but, with the 5′-end labeled oligonucleotide, two cleavage products were detected at each cleavage site. The more slowly migrating fragments presented the same electrophoretic mobilities as the fragment generated by chemical cleavage according to the G + A reaction of Maxam and Gilbert [11].

In order to determine unambiguously the nature of these 3′-termini, the radioactive bands corresponding to the more slowly migrating fragment were eluted out, the gel and aliquots were digested with T4-polynucleotide kinase in conditions such that this enzyme displays a 3′-phosphatase activity. Figure 3B

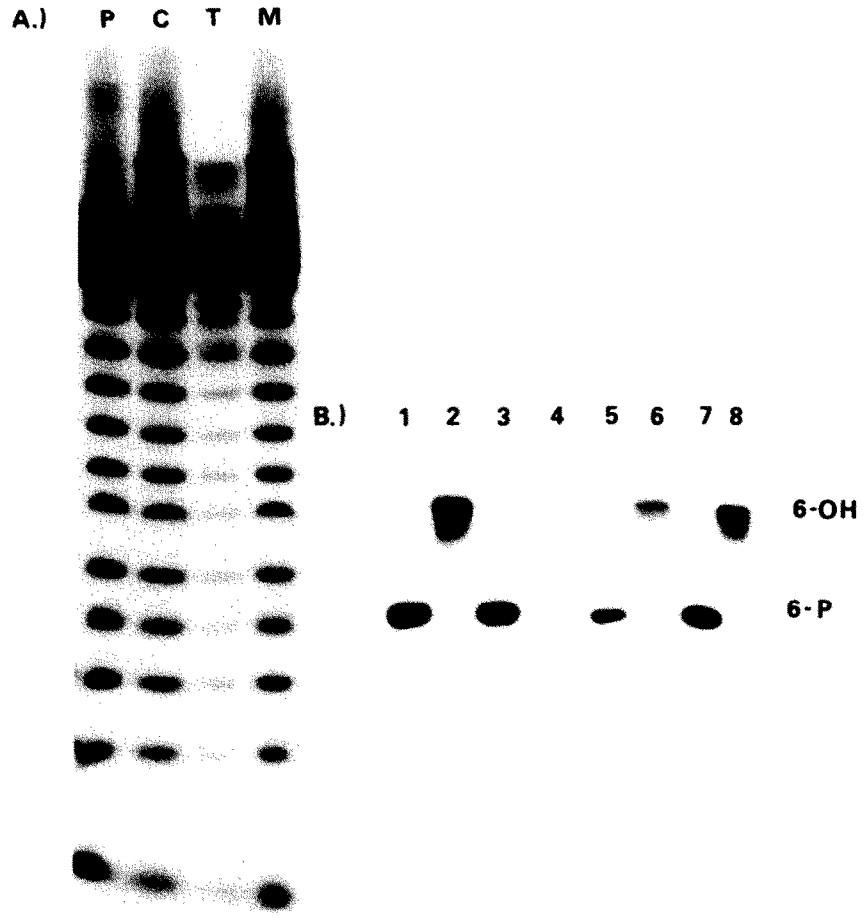

Fig. 2. (A) Polyacrylamide (20%) gel electrophoresis analysis in denaturating conditions of 3′-end labeled oligo $(dA)_{13}$ after irradiation in the presence of (P) PZ, (C) CPZ, (T) TFPZ and (M) MTPZ. (B) Polyacrylamide gel electrophoresis analysis in denaturating conditions of 6 bases long fragment generated by the PZD + near u.v. light treatment (lanes 1 and 2, PZ; lanes 3 and 4, CPZ; lanes 5 and 6, TFPZ; lanes 7 and 8, MTPZ) before (lanes 1, 3, 5 and 7) or after (lanes 2, 4, 6 and 8) digestion with CIP.

shows the comparison of the electrophoretic mobilities of 3′-phosphatase treated and untreated suspected 3′-phosphate group terminated 6 base long fragments on polyacrylamide gel in denaturating conditions. Clearly, this treatment leads to a decrease in the electrophoretic mobilities of fragments as compared to the untreated fragment. On the other hand, the faster migrating fragment could be suspected to be terminated by a 3′ phosphoglycolate group which formation has been demonstrated with hydroxyl radicals and other radiomimetic agents [14, 17]. Experiments were thus carried out in order to demonstrate unambiguously the nature of these 3′-termini. The radioactive bands corresponding to the faster migrating fragment were eluted out, the gel and aliquots were digested with ExoIII. Figure 3C shows the comparison between polyacrylamide gel electrophoresis of ExoIII treated and ExoIII untreated 4 base long fragment. This treatment leads to the formation of fragments which can be interpreted to be the results of progressive digestion. Firstly, the phosphoglycolate termini were transformed into hydroxyl termini. Thereafter, the ExoIII digested the oligonucleotide leading to the 3 base long fragment terminated by a hydroxyl group, the digestion progresses base per base. Thus, "PZD + h-induced" DNA cleavage led to the formation of the same termini at the 3′-side as reported for the indirect effect of γ-radiation [13–15]. However, as shown in Table 1 the two types of termini were not generated in the same amounts (Table 1).

DISCUSSION

From the results presented above, it clearly appears that "PZD + near u.v. light" treatment of DNA leads to chain breakage almost regardless of the nucleotide sequence and generating 5′-phosphate, 3′-phosphate and probably 3′-phosphoglycolate termini.

The DNA strand breakage occurs almost with the same frequency at each base residue, but with a slim preferency for purines. This phenomenon could be

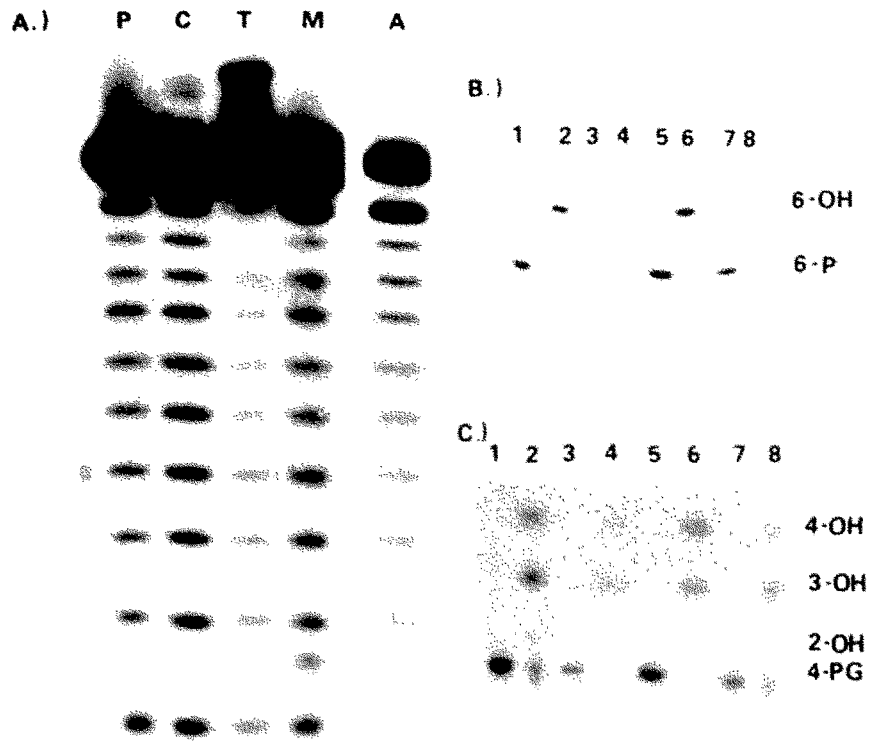

Fig. 3. (A) Polyacrylamide (20%) gel electrophoresis analysis in denaturating conditions of 5′-end labeled oligo $(dA)_{13}$ after irradiation in the presence of (P) PZ, (C) CPZ, (T) TFPZ and (M) MTPZ. (B) Polyacrylamide gel electrophoresis analysis in denaturating conditions of 6 base long fragment generated by the PZD + near u.v. light treatment (lanes 1 and 2, PZ; lanes 3 and 4, CPZ; lanes 5 and 6, TFPZ; lanes 7 and 8, MTPZ) before (lanes 1, 3, 5 and 7) or after (lanes 2, 4, 6 and 8) digestion with PNK in conditions where this enzyme shows 3′-phosphatase activity. (C) Polyacrylamide gel electrophoresis analysis in denaturating conditions of 6 bases long fragment generated by the PZD + near u.v. light treatment (lanes 1 and 2, PZ; lanes 3 and 4, CPZ; lanes 5 and 6, TFPZ; lanes 7 and 8, MTPZ) before (lanes 1, 3, 5 and 7) or after (lanes 2, 4, 6 and 8) digestion with Exo III.

Table 1. Amounts of 3′-phosphoryl- and 3′-phosphoglycolate termini generated upon PZD + hν-induced DNA breakage

		3′-P	3′-PG	% 3′-P	% 3′-PG	M 3′-P	M 3′-PG
	6	14,573	4,525	76	24		
PZ	5	14,000	5,699	71	29	72	28
	4	12,995	5,207	71	29		
	6	21,407	3,411	86	14		
CPZ	5	21,818	3,950	85	15	86	14
	4	20,794	2,395	90	10		
	6	9,754	3,190	75	25		
TFPZ	5	10,097	2,673	79	21	76	24
	4	9,409	2,577	78	22		
	6	19,345	5,373	78	22		
MTPZ	5	20,702	4,582	82	18	80	20
	4	18,866	4,398	81	19		

Oligo $(dA)_{12}$ was irradiated in the presence of PZD And the products of the reactions were separated by polyacrylamide gel electrophoresis. The radioactive bands were cut out, the gel and Cherenkov counted.

In the first column are given the origin and the length of fragments. In the other columns, 3′-P and 3′-PG refer to the radioactive counting of 3′-phosphate or 3′-phosphoglycolate bands, % 3′-P and % 3′-PG refer to the percentage of 3′-phosphate and 3′-phosphoglycolate obtained. In the M 3′-P and M 3′-PG are given the mean values of 3′-phosphate and 3′-phosphoglycolate obtained.

a reflect of an intercalation selectivity near purine residues and thus, reactive intermediates should be generated in a higher concentration near these intercalation sites.

In a previous work [10], we have demonstrated that the intermediate responsible for DNA breakage induced by PZD photoreactions was mainly the hydroxyl radical. Near u.v. light irradiation of PZD causes ionization of the drug molecule with cation radical formation and electron ejection. By a reaction with molecular oxygen, this electron forms superoxide anion which can, in the presence of DNA, be converted into hydroxyl radical, the well known DNA breaker. In addition, chlorine and/or promazinyl radicals, resulting from the photocleavage of CPZ molecule, and the cation radical of MTPZ, have also been demonstrated to be able to induce DNA breaks.

The mechanism by which γ-radiation generated hydroxyl radical induces DNA breakage is well documented ([18] and references therein). Under aerobic conditions, the primary event of the reaction leading to direct DNA breakage is a hydrogen abstraction at the C-5′ position of the deoxyribose. The resulting radical undergoes reactions with oxygen leading to sugar opening, free base and altered sugar release and free phosphate termini are generated at both sides of the breakage site. However, hydroxyl radical reaction with DNA can lead to the formation of 3′-phosphoglycolate and 5′-phosphate termini and altered base release [14, 17]. In another hydroxyl radical generating system (bleomycin-FeII-O_2), Giloni *et al.* [17] have observed that only 3′-phosphoglycolate termini are generated. These authors proposed a mechanism in which the first event is a hydrogen abstraction at the C-4′ position of the sugar, the resulting radical undergoes reactions with oxygen and reductor leading to the formation of the product.

The results we obtained seem to be quite similar to those obtained with the indirect effect of γ-radiation induced DNA breaks [13–15]. Thus, it seems reasonable to conclude that PZD photosensitized DNA breakage results from an attack of the sugar moiety of the DNA. Hydroxyl radicals which have been demonstrated to be produced through PZD irradiation, can be implicated as the main initiator species in the strand breakage reaction [10]. Moreover, the other species which has also been demonstrated to act as DNA breaker, i.e. the promazinyl-, chlorine- and MTPZ cation radical [10], should also be able to abstract hydrogen atom from the sugar moiety. This is consistent with the usual chemical reactivity of promazinyl and chlorine radical. Indeed photolysis of CPZ leads to the photoclivage of the molecule leading to the generation of these two radicals [19] and formation as final products of hydrochloric acid and promazine [20], after hydrogen abstraction from solvent molecules. In the case of the MTPZ cation radical, studies on models should be performed in order to demonstrate these possibilities.

On the other hand, as the two types of 3′-termini, 3′-phosphate or 3′-phosphoglycolate, are generated in the same amount (Table 1), it appears that the two mechanisms described above, hydrogen abstractions at the C-5′ or C-4′ positions seem to coexist. The formation of 3′-phosphoglycolate termini needs a reductor species which, in our system, could be the superoxide anion or a contaminating compound. The amount of this reductor appears to be dependent of the PZD used (Table 1). Indeed, we have previously observed that PZ and MTPZ (nothing is known about TFPZ) were more active as superoxide anion producers than CPZ [10]. Thus, in the case of PZ and MTPZ, the medium could be more reductor and so lead to more 3′-phosphoglycolate terminus formation.

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